STUDIES ON THE HEXAPOD NERVOUS SYSTEM. VI. VENTRAL NERVE CORD SHORTENING: A METAMORPHIC PROCESS IN GALLERIA MELLONELLA (L.) (LEPIDOPTERA, PYRALLIDAE)[1]

RUDOLPH L. PIPA

Department of Entomology and Parasitology, University of California, Berkeley 4, California

One of many spectacular occurrences during the metamorphosis of holometabolous insects is the transformation of the larval central nervous system into that of the adult. An account of the gross features of the change as it occurs in certain Lepidoptera was given in the last century (Newport, 1832, 1834). The salient characteristics noted include: a concentration of the mesothoracic, metathoracic, and first two abdominal ganglia; a shortening of connectives between the brain and subesophageal ganglion, and between the prothoracic and mesothoracic ganglia. In *Papilio urticae* L., under the ambient temperatures employed, these changes were realized during the first 58 hours after pupation. This contrasted with observations on *Sphinx ligustri* L., where nearly six months of developmental arrest (diapause) intervene between the most active phases of the phenomenon.

Brandt's (1879) extensive comparative study partly confirmed Newport's findings, but called attention to departures from the above pattern of reorganization in different lepidopteran species. He also depicted a fusion of the last three abdominal ganglia, an event undescribed by Newport.

The prospect of using the greater wax moth, *Galleria mellonella* (L.), in an extended analysis of neurometamorphosis has prompted the following investigation of the shortening process. Concomitant cytological features will be reported in a future communication.

Methods

Stock cultures of *Galleria* were maintained by placing several adult males and females in a screen-topped gallon jar containing a larval diet mixture of 1200 ml. Gerber Mixed Cereal, 100 ml. honey, 100 ml. glycerin, and 50 ml. water. These were kept in darkness in a constant temperature cabinet (32–35° C.). Each culture was divided at least once and replenished with fresh diet to circumvent undesired effects of crowding. Last-instar larvae were gathered from cocoons spun along the sides of the containers.

To ascertain deviations in nerve cord length due to variations in body size, last-instar larvae were separated into two groups. Individuals of one group weighed 170–190 mg., those of the other, 200–250 mg. Because values obtained from the two groups were in reasonable accord, they have been combined for statistical treatment.

Ten to 15 larvae were routinely placed in a 105 × 20 mm. plastic Petri-type culture dish, provided with cardboard tents, and maintained at a uniform tempera-

[1] This study was supported by National Institutes of Health Grant No. B-3845.

ture (30–33° C.). By the end of a day the majority had enclosed themselves within cocoons beneath the tents. The ends of each cocoon were opened at 8–12-hour intervals to permit inspection for signs of impending pupation, which are described below. Larvae demonstrating such signs were removed from their cocoons, and placed in separate dishes also kept at 30–33° C. The time during which they cast their exuviae, thus revealing "white" (untanned) pupae, was known precisely, or else approximated to within three hours.

Ventral nerve cords in desired stages of development were exposed by dorsal dissections of living insects pinned to the bottoms of Syracuse watch glasses partly filled with paraffin. The animals were immersed in Yeager's (1939) insect magnesium saline during the operation. Interganglionic connectives were measured to the nearest 0.1 mm. at a magnification of 25 diameters by means of an ocular micrometer inserted in a dissecting microscope (Wild-Greenough). Particular care was taken to minimize stretching the cords beyond their rest lengths. Measurements were routinely completely 40–50 minutes after dissection was initiated. Comparative measurements made on the same connectives at the beginning and end of this period usually differed by less than 10%.

Measurements of nerve cords from pupae and adults reared from last-instar larvae kept at 30–33° C. and 70% relative humidity (R. H.) over KOH (Buxton, 1931) did not differ significantly from those made on individuals of comparable developmental stage maintained at the same temperature, but at ambient R. H. Consequently, no attempt was made to control this factor.

Developmental Stages

All systems of a multicellular organism do not complete differentiation simultaneously. This, and the fact that developmental transitions are gradual, rather than saltatory, necessitates that selection and definition of stages be arbitrary. One criterion which might be used is the ability of the larva to spin a cocoon. As indicated by Piepho (1950) and Wiedbrauck (1955) this appears to be under hormonal control, a decrease in juvenile hormone concentration, such as occurs at the outset of pupation (Gilbert and Schneiderman, 1961; Williams, 1961), resulting in cessation of spinning.

During the present investigation an event which precedes total loss of spinning activity was detected. This involves the ability of last-instar larvae to protract and retract their anal legs, or postpedes, when these are touched. Because the reflex is lost 19–27 hours prior to ecdysis, it has served as a useful external sign of incipient pupation. The change does not seem to interfere with locomotion, nor with the larva's ability to right itself when placed in a supine position.

Last-instar larvae which have constructed cocoons, which can move their postpedes, and which can re-spin shall be referred to as stage I larvae. Stage II larvae lack the ability to protract and retract their postpedes. At the outset of this stage they can locomote readily, but this capability is soon lost. Spinning activity also ceases during this period. Stage III insects cannot walk, nor can they right themselves. They twist their abdomens vigorously from side to side, an activity which aids in shedding the larval exuviae. Stage III includes the "pharate" ("cloaked") pupal stage (Hinton, 1958). It terminates at ecdysis with the uncovering of the "white" pupa.

TABLE 1—*Mean lengths and standard deviations (millimeters) of ventral nerve cord segments in Galleria mellonella (L.) during progressive developmental stages*

Stage or Hours	SE–I	I–II	II–III	III–1	1–2	2–3	3–4	4–5	5–6	6–7, 8	6+7, 8	II+III +1+2
Stage I Number	0.1 ±0.05 30	1.1 ±0.11 30	1.4 ±0.10 30	0.4 ±0.06 30	1.1 ±0.10 30	1.2 ±0.09 30	1.4 ±0.11 30	1.4 ±0.11 30	1.4 ±0.12 30	0.9 ±0.09 30	1.8 ±0.15 20	4.3 ±0.22 20
Stage II Number	0.1 ±0.05 9	1.0 ±0.09 9	1.2 ±0.14 9	0.4 ±0.08 9	0.9 ±0.12 9	1.2 ±0.08 9	1.4 ±0.10 9	1.4 ±0.11 9	1.3 ±0.07 9	0.8 ±0.1 9	1.6 ±0.14 9	3.7 ±0.31 9
Stage III Number	0.1 ±0.05 11	0.8 ±0.10 10	1.0 ±0.13 11		0.7 ±0.17 11	1.1 ±0.07 11	1.3 ±0.07 11	1.3 ±0.08 10	1.3 ±0.09 10	0.6 ±0.06 10	1.5 ±0.08 10	3.2 ±0.28 11
0–5 Number	0.1 ±0.05 11	0.7 ±0.09 11	0.8 ±0.16 11	—	—	0.9 ±0.10 11	1.2 ±0.11 11	1.3 ±0.12 11	1.3 ±0.11 11	0.6 ±0.10 11	1.4 ±0.14 11	2.7 ±0.20 11
6–11 Number	0.1 ±0.05 13	0.6 ±0.07 13	0.7 ±0.09 13	—	—	0.9 ±0.10 13	1.1 ±0.09 13	1.3 ±0.11 13	1.3 ±0.08 13	0.4 ±0.12 12	1.1 ±0.16 11	2.4 ±0.15 11
12–17 Number	0.2 ±0.05 13	0.5 ±0.04 12	0.6 ±0.09 13	—	—	0.8 ±0.16 *1.3 ±0.06 9 *13	1.1 ±0.11 13	1.3 ±0.08 13	1.2 ±0.18 13	—	1.0 ±0.10 13	2.6 ±0.19 **2.0 ±0.11 11 **7
18–23 Number	0.3 ±0.07 10	0.4 ±0.06 11	0.5 ±0.1 11	—	—	0.7 ±0.11 *1.4 ±0.13 8 *10	1.1 ±0.09 11	1.2 ±0.13 11	1.2 ±0.12 11	—	0.9 ±0.14 10	2.3 ±0.18 **1.7 ±0.12 8 **9
24–29 Number	0.3 ±0.08 12	0.4 ±0.08 12	0.4 ±0.07 11	—	—	0.8 ±0.11 *1.5 ±0.19 6 *11	1.1 ±0.10 11	1.2 ±0.08 12	1.2 ±0.13 11	—	0.8 ±0.18 11	2.3 ±0.08 **1.5 ±0.15 11 **4
30–35 Number	0.5 ±0.05 11	0.4 ±0.09 12	—	—	—	*1.8 ±0.16 *11	1.2 ±0.08 11	1.4 ±0.08 11	1.3 ±0.10 11	—	0.6 ±0.03 12	**1.2 ±0.09 **12
36–41 Number	0.4 ±0.06 11	0.3 ±0.07 11	—	—	—	*2.1 ±0.11 *12	1.2 ±0.09 11	1.3 ±0.13 10	1.4 ±0.18 10	—	0.5 ±0.05 8	**1.1 ±0.08 **9
42–47 Number	0.5 ±0.08 10	0.3 ±0.07 10	0 10	—	—	*2.2 ±0.18 *10	1.1 ±0.10 10	1.3 ±0.13 10	1.4 ±0.14 10	—	0.6 ±0.05 10	**1.0 ±0.07 **10
48–53 Number	0.5 ±0.05 9	0.3 ±0.06 9	0 9	—	—	*2.3 ±0.08 *9	1.1 ±0.07 8	1.2 ±0.10 9	1.4 ±0.10 9	—	0.5 ±0.07 9	**1.0 ±0.04 **9
Adult ♀ <1 day old Number	0.8 ±0.09 12	0.4 ±0.05 12	0 12	—	—	*2.7 ±0.13 *12	1.3 ±0.14 12	1.6 ±0.10 12	2.0 ±0.26 12	—	0.6 ±0.03 12	**1.0 ±0.06 **12
Adult ♂ <1 day old Number	0.8 ±0.09 11	0.5 ±0.15 12	0 12	—	—	*2.8 ±0.14 *12	1.2 ±0.11 12	1.3 ±0.11 12	1.4 ±0.16 12	—	0.5 ±0.04 12	**0.9 ±0.05 **12

* Posterior border of coalescing white mass to anterior edge of third abdominal ganglion. ** Anterior border of mesothoracic ganglion to posterior edge of coalescing white mass.

Stage of pupal development was defined by the number of hours after ecdysis. Pupal age-class intervals of five hours were used throughout (Table I). An intermediate time for each interval is designated on the abscissas of Figures 2 and 3. Thus, individuals in the age class 0–5 hours are considered 3 hours old; those in the age class 6–11 hours, 9 hours old; etc. Stage II precedes ecdysis by 19–27 hours (25 of 33 cases recorded); stage III by 6–15 hours (18 of 23 cases recorded).

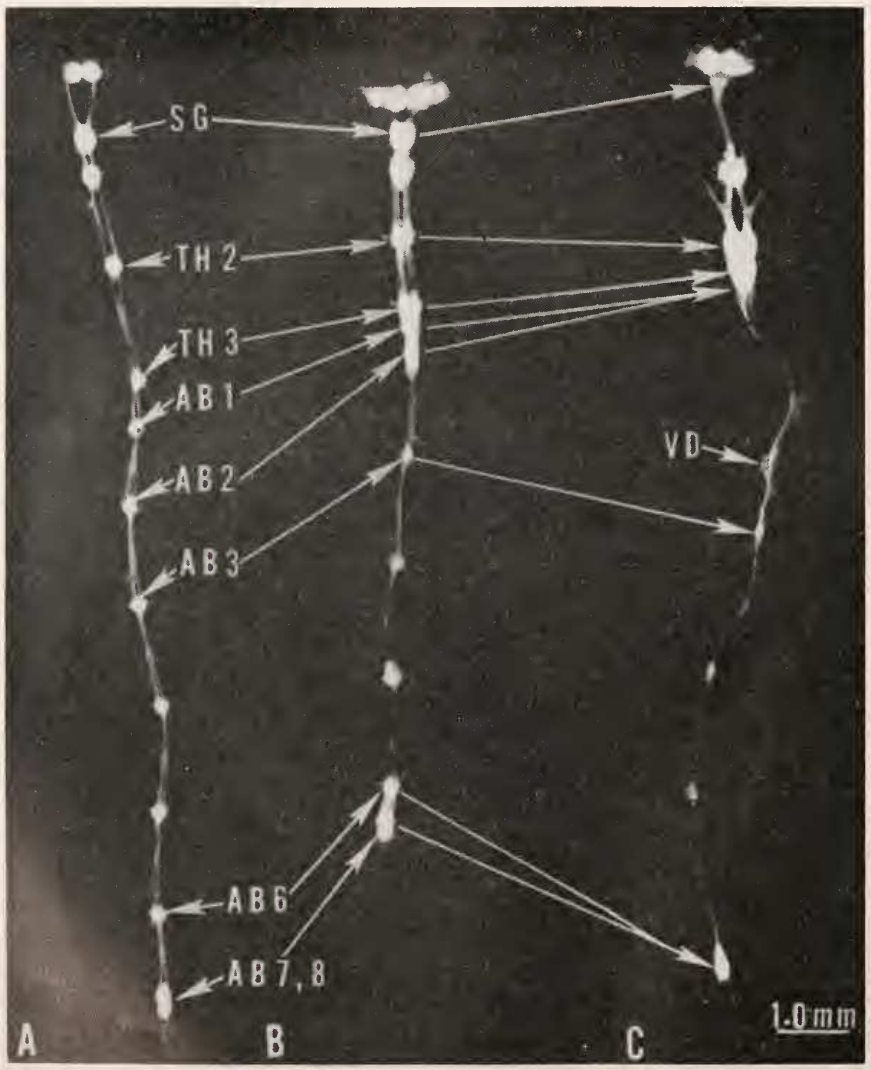

FIGURE 1. Dorsal view of central nervous systems of *Galleria*. Fixed *in situ* with 95% ethanol, removed, and preserved in 10% formalin. (A) Stage I larva; (B) Pupa, 12–15 hours after ecdysis; (C) Adult female. AB1, AB2, AB3, AB6, AB7,8, Abdominal ganglia; SG, Subesophageal ganglion; TH2, TH3, Thoracic ganglia; VD, Ventral diaphragm.

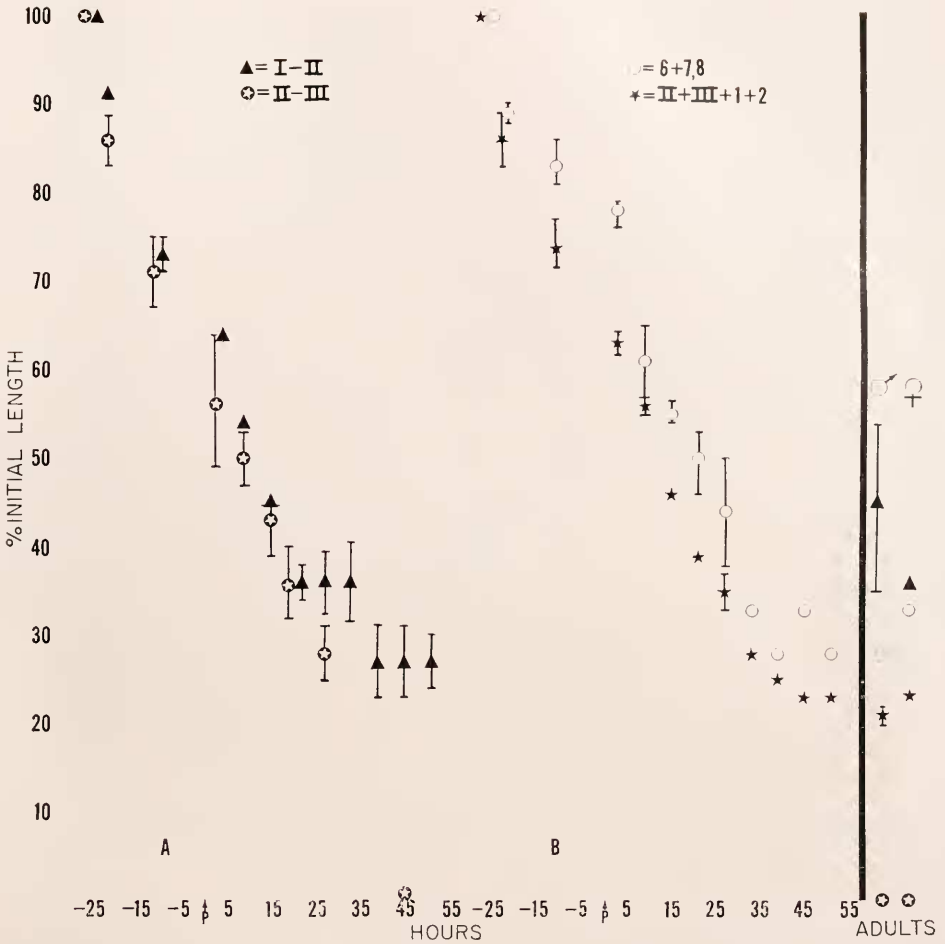

FIGURE 2. Per cent of mean initial (stage I) lengths remaining at progressive developmental stages. Standard deviations indicated by vertical lines, except where obscured by a symbol. (A) I–II, connectives between first and second thoracic ganglia; II–III, connectives between the second and third thoracic ganglia; (B) 6 + 7,8, length from anterior border of abdominal ganglion six to posterior border of abdominal ganglion eight; II + III + 1 + 2, length from anterior border of mesothoracic ganglion to the posterior border of the second abdominal ganglion. P, time of ecdysis. Percentages of stage I lengths remaining in adult males and females included at the extreme right.

Accordingly, data for stage II insects are plotted at −23 hours; those for stage III at −11 hours. Data for stage I are plotted at −26 hours, regardless of the fact that many of these insects were probably further removed from ecdysis than this.

PATTERN OF REORGANIZATION

The ventral nerve cord of a stage I larva (Fig. 1A) consists of twelve definitive ganglia, all fairly uniform in size. The first eleven are associated by

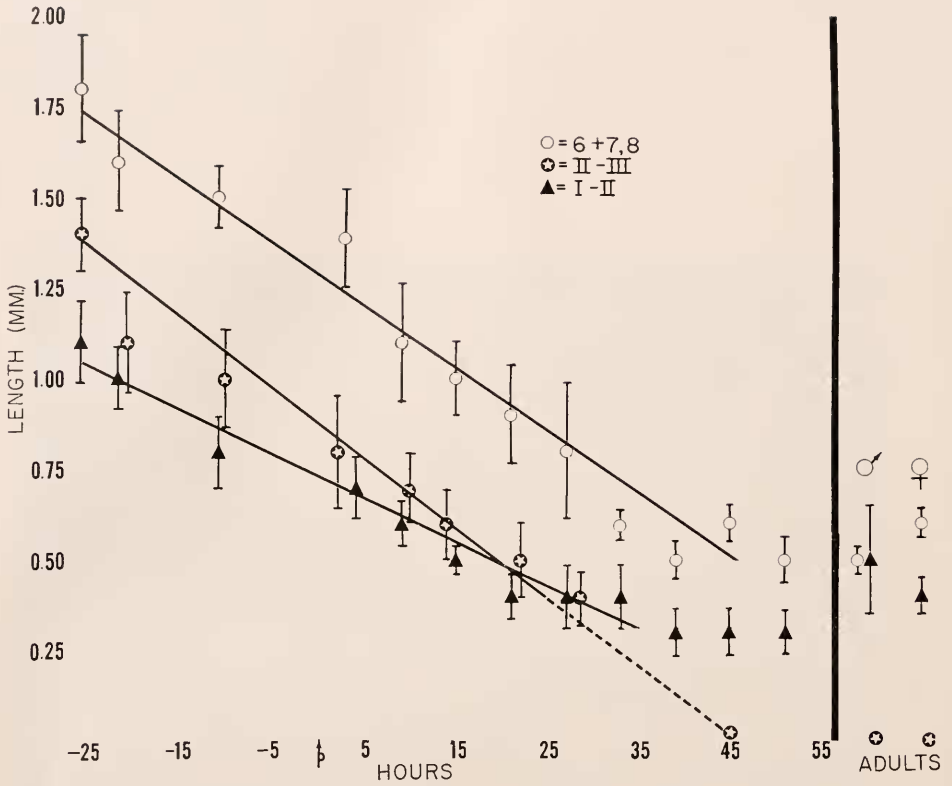

FIGURE 3. Mean lengths of selected segments of the ventral nerve cord during progressive developmental stages. Values for adult males and females are included at the extreme right. Standard deviations indicated by vertical lines. Symbols as in Figure 2.

paired connectives which vary in length. These are visible at low magnifications. The seventh and eighth abdominal ganglia, however, are contiguous. The ventral nerve cord is associated with the brain by a pair of circumesophageal connectives.

Comparison with the ventral nerve cord of a newly emerged adult (Fig. 1C) reveals prominent differences. The adult possesses nine externally recognizable ventral ganglia; the first, second, and sixth abdominal ganglia of the larva have vanished. The paired nature of the connectives between the subesophageal and first thoracic ganglia and between abdominal ganglia is no longer distinct. The second and third thoracic ganglia, lacking intervening connectives, are now contiguous. The connectives between the subesophageal ganglion and brain, and between the first and second thoracic ganglia, have also shortened; those between the subesophageal and first thoracic ganglia have lengthened. There is a striking disparity in comparative volumes of thoracic and abdominal ganglia, a reflection of disharmonic growth along the cranio-caudal axis. Another difference is that the adult abdominal interganglionic connectives are attached to the ventral diaphragm (VD, Fig. 1C), which, in turn, inserts on the abdominal exoskeleton. Contrac-

tions of muscle fibers in the diaphragm cause the abdominal nerve cord to lash from side to side within the hemocoele.

Sequential measurements of the connectives located between the first and second (I–II) and second and third (II–III) thoracic ganglia indicate that they lose 35–45% of their mean initial lengths before ecdysis (Fig. 2A). By 30–40 hours after ecdysis connectives I–II shorten maximally, losing about 75% of their original length. This contrasts with connectives II–III which continue to diminish until *ca.* 45 hours, when they disappear, leaving the ganglia contiguous.

It might be suspected that decrease in the length of I–II is not due to shortening, but is simply a result of overgrowth by the first and second thoracic ganglia. Such an interpretation is not supported by comparative measurements. These show that no more than half the 0.6–0.7 mm. lost could possibly be accounted for in this manner, and then only if all growth were directed toward I–II.

Shortening of I–II and lengthening of connectives between the subesophageal and first thoracic ganglia (SE–I) are not in phase. By 12–17 hours after ecdysis, when elongation of SE–I is first detectable, shortening of I–II is approximately 75% complete (Table I). Partial temporal separation of these two morphogenic processes occurs elsewhere within the ventral nerve cord, and shall be mentioned again below.

Reorganization, which results in disappearance of the first, second, and sixth abdominal ganglia, is indicated by Figure 1B. The first and second abdominal ganglia approach the third thoracic anteriorly, while the sixth meets the contiguous seventh and eighth posteriorly. During shortening the intervening connectives increase in diameter until they are nearly as wide as the ganglia which are about to coalesce. The discreteness of the connectives involved is lost before shortening is concluded. This has necessitated measuring from the anterior border of one ganglion to the posterior border of another. Shortening which results in concentration of the first two abdominal and the second and third thoracic ganglia was approximated by measuring from the anterior border of the second thoracic ganglion to the posterior border of the second abdominal ganglion (II + III + 1 + 2, Table I; Fig. 2B). Shortening, which results in coalescence of abdominal ganglia six, seven, and eight, was followed by measuring from the anterior border of six to the posterior border of eight (6 + 7,8 Table I; Figs. 2B; 3).

The entire second abdominal ganglion does not coalesce with the fused metathoracic-first abdominal complex. Instead, anterior migration of the definitive second abdominal ganglion ceases 12–17 hours following ecdysis (Fig. 1B). After that time an opaque white mass, presumably consisting of internal components of the second abdominal ganglion, continues forward. As this proceeds the second abdominal ganglion diminishes until an inconspicuous "hull" remains. The latter subsequently moves back as connectives between it and the advancing white mass elongate. The "hull" disappears 30–35 hours after ecdysis. Shortening, as determined by measuring II + III + 1 + 2 (Table I), is about 70% complete 12–17 hours after ecdysis, when elongation of conectives between the "hull" and white mass first becomes apparent.

Abdominal interganglionic connectives 2–3, 3–4, 4–5, and 5–6 do not shorten appreciably. Application of the *t*-test for significance between the highest and lowest means obtained for each during metamorphosis yielded *t*-values of 0.08, 0.16, 0.11, and 0.10, respectively.

Events resulting in coalescence of the sixth abdominal ganglion with seven and eight resemble those noted during fusion of the first two abdominal ganglia with the third thoracic. Thus, it appears that the contents, but not the "hull" of ganglion 6 become incorporated with 7,8. As connectives 6–7,8 shorten they increase in diameter, and it becomes increasingly difficult to separate boundaries of the ganglia (Fig. 1B). Consecutive measurements of 6 + 7,8 reveal that coalescence is completed 30–40 hours after ecdysis (Figs. 2B, 3).

When the mean lengths of selected segments of the ventral nerve cord are plotted against developmental time, the distributions depicted in Figure 3 are obtained. The origins and slopes of three of these were estimated by assuming linearity, and by applying the method of least squares. Extent of shortening per 24 hours approximated in this manner is 0.27 mm. for connectives I–II ($Y = 1.024 + 0.0112X$); 0.46 mm. for II–III ($Y = 1.37 + 0.0192X$); and 0.41 mm. for 6 + 7,8 ($Y = 1.73 + 0.0171X$). Tests for homogeneity of regression on each of the three combinations of the three lines were significant in each case (I–II vs. II–III, $t = 11.56$***; I–II vs. 6 + 7,8, $t = 9.96$***; and II–III vs. 6 + 7,8, $t = 2.38$*).

Discussion

The consecutive measurements made during the course of this study clearly demonstrate that ganglionic concentration is accomplished by shortening of intervening connectives. The explanation proposed by Murray and Tiegs (1935) for ganglionic concentration in the beetle, *Calandra oryzae*, namely, that it is due to a "proliferation of cells" and subsequent overgrowth, cannot be accepted for *Galleria*. The present study does not indicate which of the nerve cord components is responsible for decrease in length of the connectives, but the conclusion that it is caused by extraneuronal cellular migration, neuron shortening, or both, seems unavoidable.

The extent to which different sectors of the ventral nerve cord shorten is variable. The connectives between the second and third, third and fourth, fourth and fifth, and fifth and sixth abdominal ganglia do not shorten significantly. Connectives between the first and second thoracic ganglia shorten approximately 75% of their mean initial length, while those between the second and third disappear. Connectives between the third thoracic and first abdominal ganglia, first abdominal and second abdominal ganglia, and abdominal ganglia 6 and 7,8 not only disappear, but their associated ganglia coalesce. Thus, the following gradations prevail: (1) No significant shortening. (2) Partial shortening. (3) Complete shortening with establishment of contiguity between centers. (4) Complete shortening with coalescence of centers.

Gross features predict extensive concomitant modifications at the tissue and cellular level during shortening. The composition and fate of the ganglionic constituents of the advancing white mass and the shortening connectives proper are but a few of the manifestations which require histological and cytological clarification.

Ventral nerve cord shortening, well under way before ecdysis, is concluded 30–45 hours after. The gross features of neurometamorphosis described here require approximately three days for completion, or approximately 33% the mean total time from onset of pupation (stage II) to adult emergence.

Much of the shortening which occurs is compensated by subsequent elongation. Extent of shortening of connectives I–II and extent of elongation of connectives between the subesophageal and first thoracic ganglia are nearly identical (Table I). Little, if any, decrease in total ventral nerve cord length results. Similarly, loss of interganglionic connectives between the third thoracic and first abdominal ganglia, and between the first and second abdominal ganglia is entirely compensated by elongation between the second and third abdominal ganglia.

The resultant effect is an adult ventral nerve cord only slightly shorter than that of the stage I larva from which it has developed. Of greater significance, perhaps, is the correlation between the gross structural reorganization of the ventral nerve cord and the skeleto-muscular system. During metamorphosis the thoracic and first two abdominal metameres are brought closer together, the prothoracic, metathoracic, and first two abdominal segments suffering extensive reduction during the process. Shortening of the corresponding interganglionic connectives is in accord with these changes.

Statistical analysis of these data is principally due to the generous efforts of Dr. Howell V. Daly. I thank Dr. Roderick Craig and Dr. Howell V. Daly for critically reading the manuscript, and for relevant suggestions. The technical assistance of Mrs. Nancy Luykx is also gratefully acknowledged.

Summary

1. Gross features of interganglionic connective shortening during metamorphosis of *Galleria mellonella* (L.) are described. Gradations range from no significant shortening, to partial shortening, to shortening with establishment of contiguity between ganglia, to complete shortening with coalescence of ganglia.

2. Under the experimental conditions employed, shortening commences about a day prior to ecdysis, and is completed 30–45 hours after ecdysis. The rates at which various connectives shorten differ significantly from one another. If linearity is assumed, these range from 0.3 to 0.5 mm. per day.

3. Much of the shortening is compensated by subsequent elongation of connectives. The two morphogenic processes are not in phase; shortening is 70–75% complete before elongation can be detected.

4. The adult ventral nerve cord is about 15–20% shorter than that of the stage I larva from which it has developed. Shortening has altered the relative locations of certain of the ganglia so that they are in accord with structural reorganization of the skeleto-muscular system. Not only are ganglia retained close to their effector organs as a consequence, but conduction times between certain of the centers would be expected to be reduced.

LITERATURE CITED

Brandt, E., 1879. Vergleichend-anatomische Untersuchungen über das Nervensystem der Lepidopteren. *Horae Soc. Entomol. Ross.*, **15**: 1–16.

Buxton, P. A., 1931. The measurement and control of atmospheric humidity in entomological problems. *Bull. Ent. Res.*, **22**: 431–457.

Gilbert, L. I., and H. A. Schneiderman, 1961. The content of juvenile hormone and lipid in Lepidoptera: sexual differences and developmental changes. *Gen. Comp. Endocrin.* **1**: 453–472.

Hinton, H. E., 1958. Concealed phases in the metamorphosis of insects. *Sci. Prog.*, **182**: 260–275.

Murray, F. V., and O. W. Tiegs, 1935. The metamorphosis of *Calandra oryzae*. *Quart. J. Micr. Sci.*, **77**: 405–495.

Newport, G., 1832. On the nervous system of the *Sphinx ligustri*, Linn., and on the changes which it undergoes during a part of the metamorphosis of the insect. *Phil. Trans. Roy. Soc. London*, **122**: 383–398.

Newport, G., 1834. On the nervous system of the *Sphinx ligustri*, Linn., (Part II) during the latter stages of its pupa and its imago state; and on the means by which its development is effected. *Phil. Trans. Roy. Soc. London*, **124**: 389–423.

Piepho, H., 1950. Hormonale Grundlagen der Spinntätigkeit bei Schmetterlingsraupen. *Zeitschr. Tierpsychol.*, **7**: 424–434.

Wiedbrauck, J., 1955. Vom Spinnen bei Schmetterlingsraupen und seiner Abhängigkeit von Metamorphosehormonen. *Zeitschr. Tierpsychol.*, **12**: 176–202.

Williams, C. M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull.*, **121**: 572–585.

Yeager, J. F., 1939. Electrical stimulation of isolated heart preparations from *Periplaneta americana*. *J. Agr. Res.*, **59**: 121–137.